METHOD TO PRODUCE NOVEL POLYKETIDES

CROSS REFERENCE TO RELATED APPLICATIONS

5   This application is a continuation-in-part of US Serial No. 08/675,817 filed 5 July 1996 which claims priority under 35 USC 119(e)(1) from provisional application serial number 60/003,338 filed 6 July 1995. The contents of these applications are incorporated herein by reference.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY

10   ## SPONSORED RESEARCH

15   ## TECHNICAL FIELD

The invention relates to methods to synthesize polyketides which are novel using modified modular polyketides synthases (PKS) which cannot utilize a natural first module starter unit.

20   ## BACKGROUND ART

Modular polyketide syntheses are typified by the organization of deoxyerythronolide B synthase (DEBS) which produces β-deoxyerythronolide B (6-dEB) the parent macrolactone of the broad spectrum antibiotic erythromycin. DEBS consists of

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three large polypeptides each containing about 10 distinctive active sites. Fig. 1 shows, diagramatically, the nature of the three DEBS modules encoded by the three genes *eryAI*, *eryAII* and *eryAIII*.

Various strategies have been suggested for genetic manipulation of PKS to produce novel polyketides. New polyketides have been generated through module deletion (Kao, C.M. *et al.*, *.J. Am. Chem. Soc.* (1995) 117:9105-9106; Kao, C.M. et al, *J. Am. Chem. Soc.* (1996) 118:9184-9185). Also reported to provide novel polyketides are loss of function mutagenesis within reductive domains (Donadio, S. *et al.*, *Science* (1991) 252:675-679; Donadio, S. et al, *Proc. Natl. Acad. Sci. USA* (1993) 90:7119-7123; Bedford, D. *et al.*, *Chem. Biol.* (1996) 3:827-831) and replacement of acyl transferase domains to alter starter or extender unit specificity (Oliynyk, M *et al.*, *Chem. Biol.* (1996) 3:833-839; Kuhstoss, S. *et al.*, *Gene* (1996) 183:231-236), as well as gain of function mutagenesis to introduce new catalytic activities within existing modules (McDaniel, R. *et al.*, *J. Am. Chem. Soc.* (1997) in press). In some of these reports, downstream enzymes in the polyketide pathway have been shown to process non-natural intermediates. However, these methods for providing novel polyketides suffer from the disadvantages of requiring investment in cloning and DNA sequencing, the systems used being limited to producer organisms for which gene replacement techniques have been developed, primer and extender units that can only be derived from metabolically accessible CoA thioesters, and the fact that only limited auxiliary catalytic functions can be employed.

The DEBS system in particular has been shown to accept non-natural primer units such as acetyl and butyryl-CoA (Wiesmann, KEH et al, *Chem. Biol.* (1995) 2:583-589; Pieper, R. et al, *J. Am. Chem. Soc.* (1995) 117:11373-11374) as well as N-acetylcysteamine (NAC) thioesters of their corresponding ketides (Pieper, R. *et al.*, *Nature* (1995) 378:263-266). However, it has become clear that even though such unnatural substrates can be utilized, competition from the natural starter unit has drastically lowered yield. Even if starter units are not supplied artificially, they can be inherently generated from decarboxylation of the methylmalonyl extender units employed

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by the DEBS system (Pieper, R. *et al.*, *Biochemistry* (1996) 35:2054-2060; Pieper, R. *et al.*, *Biochemistry* (1997) 36:1846-1851).

Accordingly, it would be advantageous to provide a mutant form of the modular polyketide synthesis system which cannot employ the natural starter unit. Such systems can be induced to make novel polyketides by supplying, instead, a suitable diketide as an NAC thioester or other suitable thioester. Mutations have been made in the past to eliminate the competition from natural materials (Daum, S.J. *et al.*, *Ann. Rev. Microbiol.* (1979) 33:241-265). Novel avermectin derivatives have been synthesized using a randomly generated mutant strain of the overmectin producing organism (Dutton, C.J. *et al.*, *Tetrahedron Letters* (1994) 35:327-330; Dutton, C.J. *et al.*, *J. Antibiot.* (1991) 44:357-365). This strategy is, however, not generally applicable due to inefficiencies in both mutagenesis and incorporation of the substrates.

Thus, there is a need for a more efficient system to prepare novel polyketides by inhibiting competitive production of the natural product.

DISCLOSURE OF THE INVENTION

The invention is directed to methods to prepare novel polyketides using modified modular polyketide synthase systems wherein directed modification incapacitates the system from using its natural starting material. Novel polyketides can then be synthesized by overriding the starter module and supplying a variety of suitable diketide substrates.

Thus, in one aspect, the invention is directed to a method to prepare a novel polyketide which method comprises providing a thioester diketide substrate to a modular PKS comprising at least two modules under conditions wherein said substrate is converted by said modular PKS to a product polyketide, wherein said PKS has been modified to prevent its utilization of the native starter unit. In other aspects, the invention is directed to the modified modular PKS which is disarmed with respect to utilization of the native

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starter substrate supplying the initial two carbon unit, and to suitable cells modified to contain this disarmed PKS. The invention is further directed to recombinant materials for production of the modified PKS and to the novel polyketides produced by this system.

5   ## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of the DEBS modular PKS.

Figure 2 shows the products of a modified DEBS construct wherein the ketosynthase in module 1 is disarmed.

Figure 3 shows the processing of 6-dEB derivatives to erythromycin-D derivatives.

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MODES OF CARRYING OUT THE INVENTION

The invention provides modular PKS systems which are disarmed with respect to loading the native starting material and their corresponding genes. In a particularly preferred embodiment, the ketosynthase (KS) of module 1 is inactivated so as to prevent

15   competition from the native starter unit. Other approaches to similarly disarming the PKS involve inactivating the acyl transferase (AT) or acyl carrier protein (ACP) functions of module 1.

The PKS of the invention must contain at least two modules but may contain additional modules and, indeed may, represent complete synthase systems. While the

20   DEBS PKS system is used to illustrate the invention, any modular PKS can be used, such as the modular PKS resulting in the production of avermectin, rapamycin and the like. Suitable mutations can be introduced by known site specific mutagenesis techniques.

Other micro-organisms such as yeast and bacteria may also be used. The novel polyketides may be synthesized in a suitable hosts, such as a Streptomyces host, especially

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a Streptomyces host modified so as to delete its own PKS. The polyketides may also be synthesized using a cell-free system by producing the relevant PKS proteins recombinantly and effecting their secretion or lysing the cells containing them. A typical cell-free system would include the appropriate PKS, NADPH and an appropriate buffer and substrates

5 required for the catalytic synthesis of polyketides. To produce the novel polyketides thioesters of the extender units are employed along with the thioester of a diketide.

The following examples are intended to illustrate but not to limit the invention.

Preparation A

STARTING MATERIALS

10 Streptomyces coelicolor CH999, which has been engineered to remove the native PKS gene cluster is constructed as described in WO 95/08548. pRM5, a shuttle plasmid used for expressing PKS genes in CH999 was also described in that application. Plasmid pCK7 which contains the entire DEBS modular system was described in the foregoing application as well.

Example 1

Preparation of DEBS 1+ 2+TE

A modified DEBS PKS system containing only modules 1 and 2 and thioesterase (TE) activity, designated DEBS 1+2+TE, was subjected to site directed mutagenesis to inactivate module 1 KS by replacing the active site cysteine residue in the signature sequence cys-ser-ser-ser-leu by alanine. The resulting expression plasmid, designated

20 pKAO179, encodes a 2-module PKS which is inactive under the standard reaction conditions for synthesis of the native product, *i.e.*, propionyl-CoA, methylmalonyl-CoA, and NADPH. The details of this construction are set forth in Kao, C.M. *et al*, Biochemistry (1996) 35:12363-12368. When provided with the diketide thioester (2S,

25 3R)-2-methyl-3,3-hydroxy-pentanoyl-N-acetylcysteamine thioester, and with methylmalonyl-CoA, and NADPH, the triketide product set forth below is obtained.

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The triketide product is produced under these conditions when the PKS is incubated in a cell-free system or can be duplicated *in vivo* by providing the appropriate diketide thioester analogs to actively growing cultures of CH99 containing the modified expression plasmid:

5   A culture of *S. coelicolor* CH999/pKAO179 is established by inoculation of 200 mL of SMM medium (5% PEG-800, 0.06% $MgSO_4$, 0.2% $(NH_4)_2SO_4$, 25 mM TES, pH 7.02, 25 mM KH_2PO_4, 1.6% glucose, 0.5% casamino acids, trace elements) with spores. The culture is incubated at 30°C with shaking at 325 rpm. A solution of (2S, 3R)-2-methyl-3-hydroxypentanoyl N-acetlycysteamine thioester (100 mg) and 4-pentynoic

10   (15 mg) in 1 mL of methylsulfoxide is added to the culture in three parts: after 50 hours (400 mL); after 62 hours (300 mL); and after 86 hours (300 mL). After a total of 144 hours, the culture is centrifuged to remove mycelia. The fermentation broth is saturated with NaCl and extracted with ethyl acetate (5 x 100 mL). The combined organic extract is dried over Na_2SO_4, filtered, and concentrated. Silica gel chromatography yields (2R, 3S,

15   4S, 5R)-2,4-dimethyl-3, 5-dihydroxy-n-heptanoic acid δ-lactone.

Example 2

Preparation of Polyketides from the DEBS Cluster

The active site mutated module 1 KS domain of the *eryAI* (DEBS 1 gene) is provided on plasmid pCK7, which contains the *eryAI*, *eryAII* (DEBS 2) and *eryAIII*

20   (DEBS 3 genes) under control of the *actI* promoter. Expression from this plasmid pJRJ2 results in a suitably modified full length PKS system. (Kao, C.M *et al.*, *Science* (1994) 265:409-512. pJRJ2 was transformed into CH999 and grown on R2YE medium. No detectable 6 DEB-like products were produced.

In more detail, lawns of CH999/pJRJ2 were grown at 30°C on R2YE agar plates

25   containing 0.3 mg/ml sodium propionate. After three days, each agar plate was overlayed with 1.5 mL of a 20 mM substrate solution in 9:1 water:DMSO. After an additional 4 days, the agar media (300 mL) were homogenized and extracted three times with ethyl

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acetate. The solvent was dried over magnesium sulfate and concentrated. Concentrated extracts were purified by silica gel chromatography (gradient of ethyl acetate in hexanes) to afford products.

However, when substrate 2, prepared by the method of Cane *et al.*, *J. Am. Chem. Soc.* (1993) 115:522-526; Cane, D.E. *et al.*, *J. Antibiot.* (1995) 48:647-651, shown in Figure 2 (the NAC thioester of the native diketide) was added to the system, the normal product, 6 dEB was produced in large quantities. Administration of 100 mg substrate 2 to small scale cultures (300 ml grown on petri plates as described above, resulted in production of 30 mg 6 dEB, 18% yield.

<div align="center">Example 3</div>

<div align="center">Production of Novel Polyketides</div>

Diketides with the structures shown in Figure 2 as formulas 3, 4, and 5 were then administered to growing cultures of CH999/pJRJ2 under the conditions of Example 2. Substrates 3 and 4 were prepared as described for Substrate 2 but substituting valeraldehyde and phenylacetaldehyde, respectively for propionaldehyde in the aldol reactions. The preparation of Substrate 5 was described by Yue, S. *et al.*, *J. Am. Chem. Soc.* (1987) 109:1253-1255. Substrates 3 and 4 provided 55 mg/L of product 6 and 22 mg/L of product 7. respectively. Substrate 5 resulted in the production of 25 mg/L of the 16 member lactone 8, an unexpected product.

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Example 4

Processing of the Polyketide Products

The successful processing of unnatural intermediates by the "downstream" modules of DEBS prompted an experiment to determine whether the post-PKS enzymes in the erthromycin biosynthetic pathway might also accept unnatural substrates. In the natural producer organism, *Saccharopolyspora erythrea*, 6dEB undergoes several enzyme-catalyzed transformations. Oxidation at C6 and glycosylations at C3 and C5 afford erythromycin D (formula 9 in Figure 3) and subsequent transformations afford erythromycins A, B, and C. *S. erythrea* mutant (A34) (Weber, J.M. *et al., J. Bactiol.* (1985) 164:425-433) is unable to synthesize 6dEB. This strain produces no erythromycin when grown on R2YE plates (as judged by the ability of extracts to inhibit growth of the erythromycin-sensitive bacterium *Bacillus cereus*). However, when 6dEB (which has no antibacterial activity) is added to the culture medium, extracts exhibited potent antibacterial activity.

Samples of 6dEB derivatives 6 and 7 were assayed for conversion by this strain. Partially purified extracts demonstrated inhibition of *B. cereus* growth, and mass spectrometry was used to identify the major components of the extracts as formula 10 in Figure 3 (from 6) and formula 11 (from 7).

In more detail, purified 6 and 7 (5 mg dissolved in 7.5 mL 50% aqueous ethanol) were layered onto R2YE plates (200 mL media/experiment) and allowed to dry. *S. erythrea A34* was then applied so as to give lawns. After 7 days of growth, the media were homogenized and extracted three times with 98.5:1.5 ethyl acetate:triethylamine. Pooled extracts from each experiment were dried over magensium sulfate and concentrated. Extracts were partially purified by silica gel chromatography (gradient of methanol and triethylamine in chloroform). The partially purified extracts were examined by TLC and mass spectrometry. For antibacterial activity analysis, filter discs were soaked in 400 µM ethanolic solutions of erythromycin D, 10 and 11, as well as a concentrated

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extract from *S. erythrea A34* which had been grown without addition of any 6-dEB analogs. Disks were dried and laid over freshly-plated lawns of *Bacillus cereus*. After incubation for 12h at 37°C, inhibition of bacterial growth was evident for all compounds but not for the control extract.

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